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PRINCIPAL INVESTIGATOR: Senyon Choe, Ph.D.

CONTRACTING ORGANIZATION: The Salk Institute for Biological Studies
La Jolla, CA 92037-1099

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Senyon Choe, Ph.D.

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La Jolla, CA 92037-1099

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We have completed the crystal structure determination of diphtheria toxin bound to its normal receptor (Louie et al., Molecular Cell, in press). The crystal structure of diphtheria toxin (DT) complexed 1:1 with a extracellular fragment of its cell-surface receptor reveals that a ß-sheet in the receptor-binding domain of DT packs against a ß-sheet of the EGF-like fold of the receptor, thus the interaction is primarily mediated through side chains. Such unusual interaction as the protein-protein interface provides an excellent structural basis for altering side chains to modulate the binding specificity of the toxin to other EGF-like receptors. Using a three-dimensional picture as a guide, we have identified key atoms dictating the fit between toxin and receptor. To fit to the new molecular surface provided by heregulin that is distinct from that of the natural toxin receptor, we will modify amino acids of the toxin surrounding heregulin-specific amino acids by molecular biology techniques. In subsequent years, the function of this recombinant toxin will first be tested in cell culture systems of breast cancer cell lines.

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S. Choe 9/28/97

PI - Signature Date

Table of Contents

INTRODUCTION

Our goal is to design a cytotoxic protein that recognizes and binds to a cell that produces heregulin (HRG), an activating growth factor ligand for HER-4, whose overexpression is correlated with breast cancer cells. Molecular design is based on the crystal structure of diphtheria toxin (DT) 1:1 complexed with an extracellular fragment of its receptor protein, a membrane-bound precursor of human heparin-binding EGF (HB-EGF). Diphtheria toxin is produced by the bacterium *Corynebacterium diphtheriae* lysogenized by corynephage ß (Collier, 1975). A single molecule of the toxin, a protein of 535 amino-acid residues, can be sufficient to kill a cell. The killing action of DT involves three distinct steps: (1) binding to a receptor on the surface of sensitive cells and subsequent receptor-mediated endocytosis; (2) translocation of the catalytic domain of the toxin across the endosomal membrane and into the cytoplasm of the cell, a process induced by the acidic environment inside the endosome; and (3) catalytic transfer of an ADP-ribosyl group from NAD^+ to a specific modified histidine on the ribosomal elongation factor-2, thus preventing protein synthesis in the cell and leading to cell death.

We have found that the structural scaffold of HBEGF is structurally homologous to that of HRG. The structure of the DT/HBEGF complex we have completed at 2.65 Å resolution allowed us to model the binding interface of HRG on the basis of the structure of HB-EGF (Louie et al., in press). To establish the protocol and demonstrate the principle, we chose mouse HB-EGF as a first target to switch the binding affinity. Thus we have selected an initial set of amino acids (Lys_{516} and His_{391}) of DT from the DT/receptor binding interface that define the binding specificity of human HBEGF. These selected sites have been randomized by PCR-based mutagenesis systematically and mutants are being screened for their binding affinity and specificity for mouse HBEGF. Mutagenic variants of DT selected from this screening will then be tested for its ability of delivering its cytotoxicity to mouse cells in a targeted manner. Subsequently, the same principle will be used for HRG as a target on breast cancer cell lines.

BODY OF RESULTS

Binding stoichiometry of DT and HBEGF

Complexation between DT and HB-EGF was assessed with methods that exploit the substantial overall charge difference between the two proteins (pI DT ~ 5.5, pI HBEGF ~ 10.0). Both isoelectric focusing and native electrophoresis permit the complexed proteins to be separated from the individual components. From experiments in which a fixed amount (known accurately from amino-acid analysis) of one of the proteins was titrated with varying amounts of the other, DT and HBEGF were determined to form a complex with 1:1 stoichiometry.

General features of the DT-HBEGF interaction

The receptor-binding (R) domain of DT consists of an antiparallel ß-barrel which is somewhat flattened to form two distinct ß-sheets (see Figure 1). The ß-strands have a similar overall topology to that of the immunoglobulin (Ig) domains (Choe *et al.*, 1992); the strands Rß2, 3, 4, 5, 8, 9, and 10 correspond to the A, B, C, D, E, F, and G strands of the Igs. Like the Ig variable domain, the DT R-domain contains an insertion of two ß-strands with respect to an Ig constant domain. However, the insertion occurs after the fourth strand of the barrel (Rß5) in DT, rather than after the third (C) as in the Ig variable domain.

The structure of the DT-HBEGF complex reveals that the crescent-shaped HBEGF molecule packs against a saddle-shaped crevice in the wall of the ß-barrel of the DT R-domain (Figure 1). The crevice flanks the face of the b-sheet containing the strands Rb6, 7, 4, 9, and 10. The loop connecting the latter two strands, which was earlier predicted to participate in receptor recognition (Shen *et al.*, 1994), forms a large part of the lower wall of the crevice (as viewed in Figure 1). The EGF module of HBEGF consists largely of a ß-hairpin, which in the complex contributes the third sheet to a three-layered b-sandwich. In this sandwich, the peptide-bond planes in each ß-sheet are nearly parallel to those in the adjacent sheet. The ß-strands in each sheet cross at an angle of approximately –40° with respect to strands in the adjacent sheet. The closest approach of a-carbons in the apposed ß-sheets of the two molecules is about 8-9 Å. It is notable that the close association of the major ß-hairpin of HBEGF with a ß-sheet from DT shares many features with the intramolecular interaction between the EGF and fibronectin-like modules of tissue-type plasminogen activator (Smith *et al.*, 1995). In t-PA, the same concave face of EGF participates in binding, although the interaction between the apposed ß-structures is somewhat less extensive.

Although face-to-face packing of ß-sheets is common within single protein domains (Chothia & Janin, 1981), it is rarely seen between protein molecules. However, intermolecular association of this type does occur frequently with proteins from the Ig superfamily. In particular, in terms of both the inter-sheet separation distance and ß-strand crossing-angle, the DT-HBEGF interaction bears remarkable similarity to the interaction between two constant (C_{H1}-C_L or C_{H3}-C_{H3}) domains in an Ig molecule (entries 1mco and 8fab in the PDB), or the related interaction between Ca and Cb domains of the T-cell receptor (Garcia *et al.*, 1996). This similarity is perhaps not unexpected considering the structural similarity between the DT R-domain and an Ig domain. In the dimer of Ig constant domains, a four-layered ß-sandwich is formed, and the saddle-shaped crevice is less curved to accommodate the wider ß-sheet of the partner constant domain. In comparison to the DT R-domain, the Ig constant domains interface through the opposite sheet (ABED) of the b-sandwich. However, several cell-adhesion molecules (including CD2, CD8, and VCAM-1) that are members of the Ig superfamily have been shown to use the CFG face of the ß-sandwich to interact with other adhesion proteins (Jones *et al.*, 1995).

The DT-HBEGF Interface

At the DT-HBEGF interface, each molecule buries approximately 1100 $Å^2$ of surface. For the smaller HBEGF molecule, the buried area represents nearly 50% of the total surface area. Due to the face-to-face association of ß-sheets at the interface, the main-

chain polar groups in each sheet are involved primarily in intra-sheet hydrogen bonding. As a result, the central part of the intermolecular interface involves only amino-acid side chains, and is predominantly non-polar (Figure 2). Non-polar surfaces are formed on DT by the side chains of Phe_{389}, Ala_{430}, Leu_{433}, Ile_{464}, Val_{468}, Phe_{470}, Gly_{510}, Leu_{512}, Val_{523}, and Phe_{530}; and on HBEGF by the side chains of Val_{124}, Leu_{127}, Ala_{129}, Pro_{130}, Ser_{131}, Ile_{133}, and Pro_{136}, the Gly_{137} backbone, and the Cys_{134}-Cys_{143} disulfide. An exception to the non-polarity of the core of the interface is the side chain of DT-Lys_{526}, which is hydrogen bonded to the carbonyl oxygens of HBEGF-Cys_{132} and Glu_{141}. The majority of the direct hydrogen-bond interactions (17 in total) are formed at the periphery of the interface, and a number of these involve main-chain polar groups. Additional hydrogen-bond interactions are mediated by bridging water molecules, including two networks of six water molecules occupying cavities adjacent to Lys_{526}. Particularly notable interactions are formed by the side chain of HBEGF-Glu_{141}, which bridges two basic side chains of DT, His_{391} and Lys_{516}. There are five intermolecular ion pairs and only the two small cavities (accessible to a probe sphere 1.4 Å in radius) adjacent to DT-Lys_{526}. These factors together with the direct and water-mediated interactions (Table) undoubtedly contribute to the specificity of DT for (the human form of) HBEGF and the relatively tight binding ($K_d \sim 10^{-8}$ M).

CONCLUSIONS

Similarity of HBEGF to other EGF-like proteins

The three-dimensional structures of a number of EGF modules determined, both as isolated units and as portions of a larger protein are similat to that of HBEGF in the DT-HBEGF complex, a long and short ß-hairpin stabilized by three disulfide bonds (Campbell & Bork, 1993). A superposition of HBEGF with a number of other known structures of EGF modules reveals that the conformations and relative orientation of the major and minor ß-hairpins, the hydrogen-bonding pattern within the hairpins, and the conformations of the reverse turns and of the three disulfide bonds are generally all in good agreement. Like TGF-a, HBEGF has a one-residue deletion in the reverse turn preceding the first ß-strand, but this deletion causes only a small, local perturbation in the conformation of the polypeptide chain. The largest structural differences among the various EGFs occur within the N-terminal A-loop (between the first and second cysteines), and around the B-loop reverse turn that links the two strands of the major ß-hairpin. The overall root-mean-square (r.m.s.) positional deviation of equivalent a-carbons between HBEGF and the other EGF modules is typically 1.2-1.5 Å for 31-38 residues, and is 1.26 Å for 35 residues for a-heregulin (Jacobsen *et al.*, 1996), the EGF structure with the greatest sequence similarity to HBEGF.

Comparison to DT-nonbinding Mouse HBEGF

Mouse HBEGF, which is not recognized by DT, differs from the human or monkey forms at ten sites within the EGF module. The effects of these amino-acid replacements can be assessed from simple modeling of the substitutions onto the structure of the complexed human HBEGF. Residues 125 and 135 face away from the DT molecule, and thus substitutions at these positions are unlikely to affect binding. The other substitutions do occur at the interface, and curiously, all involve replacement by a larger

side chain. Two (Lys122Arg and Ser147Thr) can likely be accommodated, whereas four others (Phe115Tyr, Val124Leu, Leu127Phe, and Ala129Thr) are certain to create at least some steric conflicts. The Ile133Lys substitution places a longer, more polar side chain into the predominantly non-polar interior of the DT-HBEGF interface. However, the Lys side chain could possibly reorient toward the periphery of the interface and form hydrogen bonds with polar groups of DT-Asp_{467}. Clearly, the most detrimental substitution is expected to be Glu141His, which would potentially place three basic side chains (His_{141} and DT's His_{391} and Lys_{516}) in close proximity in the complex. Two further observations underscore the importance of interactions formed at the site of this substitution. First, as described above, the Lys516Ala mutant shows the largest decreases in toxicity and receptor-binding affinity of any DT mutant studied (Shen *et al.*, 1994). Second, chimeras of mouse HBEGF with either the human or monkey forms show that the single amino-acid substitution Glu141His is sufficient to preclude almost all DT binding (Mitamura *et al.*, 1995). Furthermore, cells expressing monkey HBEGF with this site-directed mutation are 100-fold less sensitive to the toxin and have 12-fold reduced DT affinity relative to cells expressing wild-type HBEGF (Hooper & Eidels, 1996). Nevertheless, binding determinants besides Glu_{141} are also important, as mouse HBEGF with the single "humanizing" His141Glu mutation shows only slight binding to DT.

CURRENT DIRECTION

The strong structural similarity between HBEGF in the complex and other EGF modules both in solution and in crystals has two implications. First, the R-domain of the DT molecule has been adapted to bind HBEGF such that little distortion occurs relative to the uncomplexed form of the receptor. Second, other EGF modules will make feasible recognition targets for engineered DTs with redesigned binding specificities. Those positions surrounding the key determinants of the receptor are evaluated by measuring the binding affinity of various point mutants. Random codons have been introduced and we are in the process of screening for those that have altered binding affinity.

Figure 1. Side- and Frontview of R domain (large) and HBEGF (small).

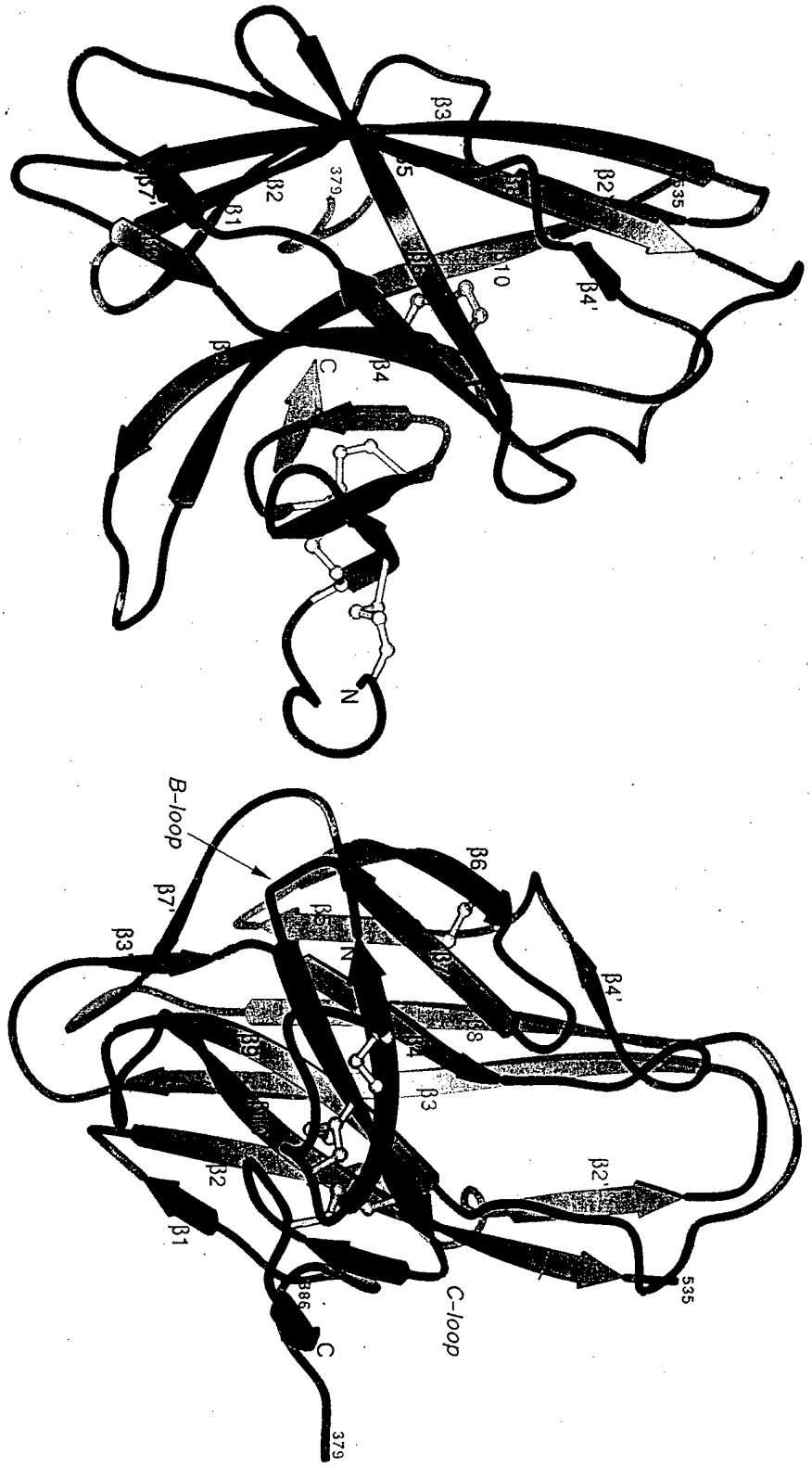

Figure 2. Details of interaction between R domain of DT and HBEGF. HBEGF residues are underlined.

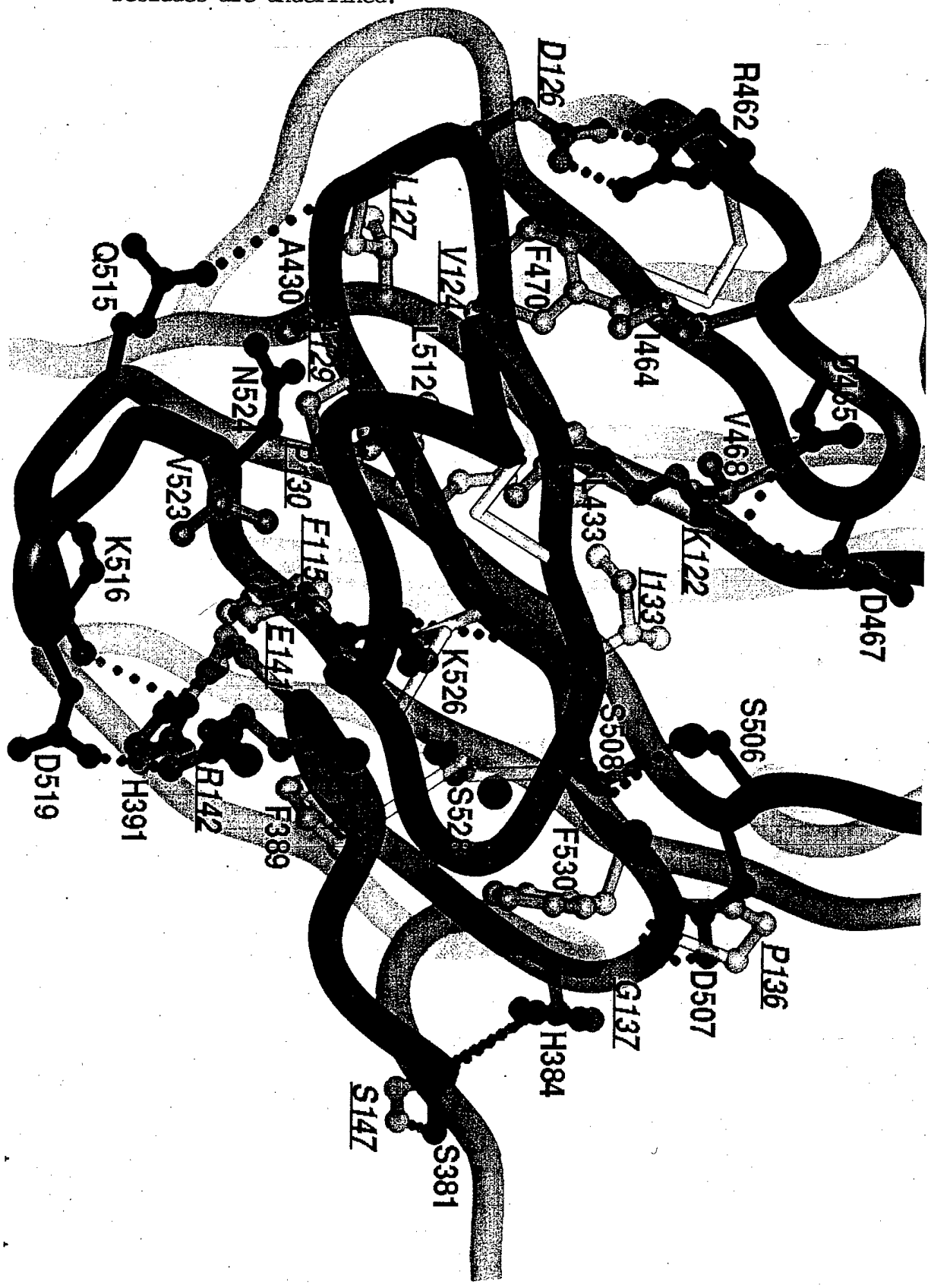

Table. Interactions between DT and HBEGF in the complex

DT atom	HBEGF atom	Distance (Å)	DT atom	HBEGF atom	Distance (Å)
Hydrogen bond and salt bridge			*Van der waals*		
Ser381 OG	Ser147 OG	2.7	Ala430 CB	Leu127 CD2	4.0
His384 NE2	Ser147 OT1	3.0	Leu433 CD1	Ser131 CB	3.7
His384 NE2	Ser147 OT2	3.7	Leu433 CD1	Ile133 CD1	3.9
His391 NE2	Glu141 OE1	2.8	Ile464 CD1	Val124 CG2	4.0
Arg462 NE	Glu126 OE1	2.9	Val468 CG2	Ile133 CD1	3.9
Arg462 NH2	Glu126 OE2	2.8	Phe470 CD1	Val124 CG1	3.9
Asp465 OD2	Lys122 NZ	2.7	Phe470 CE1	Leu127 CD1	3.9
Asp467 OD2	Lys122 NZ	3.7	Phe470 CZ	Leu127 CD1	3.9
Ser506 OG	Cys134 O	2.7	Leu512 CD1	Leu127 CD1	3.7
Asp507 OD1	Gly137 N	3.0	Asn524 OD1	Leu127 CD1	3.7
Asp507 OD2	Gly137 N	3.3	Phe530 CE2	Gly137 CA	3.8
Gln515 NE2	Leu127 O	3.3			
Lys516 NZ	Glu141 OE2	3.7			
Asp519 OD2	Arg142 NH1	3.1			
Lys526 N	Glu141 OE1	2.9			
Lys526 NZ	Cys132 O	3.1			
Lys526 NZ	Glu141 O	3.2			

Water mediated

DT atoms	HBEGF atoms	Water
Ser506 OG; Asp507 N, OD1; Ser508 OG	Cys134 O; His135 O	1
Ser508 OG; Ser528 OG	Tyr138 O	2
Ser528 OG; Lys526 O	Gly140 N	3
Lys526 O	Glu141 OE1	4
Asn524 N; Asn524 OD1	Pro130 O	5
Ser505 O	Ile133 O	6

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